PLASMIDS OF *STREPTOMYCES KASUGAENSIS* MB273: THEIR POCK FORMATION, THEIR DISPENSABLE ENDONUCLEASE CLEAVAGE SITES FOR POCK FORMATION, AND TRANSFORMATION OF *S. KASUGAENSIS* MB273 BY THEM

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(Received for publication December 6, 1983)

Plasmid-free strains of *Streptomyces kasugaensis* MB273 were isolated. In mating experiments *S. kasugaensis* MB273 was found to cause a lethal zygosis (pock) phenotype in a plasmid-free host. The pock-forming plasmids were identified as either pSK1* or pSK2* on the basis of their endonuclease cleavage-sites. The strain carrying pSK1* was found to induce pocks on the strain bearing pSK2*, and *vice versa*. The endonuclease cleavage-sites in pSK1* and pSK2* that were nonessential for pock formation were determined in deletion or insertion derivatives. The single sites for *Bcl* I and *Sal* I in pSK1* and for *Bgl* II in pSK2*, respectively, could be useful for DNA cloning without destroying pock-forming ability. Protoplasts of *S. kasugaensis* MB273-derivatives prepared in stationary phase of mycelial growth were competent for transformation, however, regeneration frequencies decreased during this phase.

Streptomyces species are important producers of secondary metabolites, including antibiotics of medical and agricultural importance as well as other biologically active substances. Application of gene cloning techniques to *Streptomyces* could lead to improved fermentation yields of their products and to the production of new secondary metabolites. Some *Streptomyces* plasmids[1~5] have been found to elicit a "lethal zygosis (pock)" phenotype and this has led to the establishment of efficient gene-cloning systems in *S. coelicolor* A3(2)[6] and *S. lividans*[3,6,7]. *S. kasugaensis* MB273 produces both kasugamycin and aureothricin and carries several small multi-copy plasmids[8]; recently, a protoplast-regeneration system has been established[9]. *S. kasugaensis* MB273 was found to cause pocks in a lawn of a plasmid-free strain of MB273. This paper describes the isolation of plasmid-free strains, the endonuclease cleavage-sites in plasmids that were nonessential for pock formation and conditions for efficient transformation of *S. kasugaensis* MB273.

Materials and Methods

Bacterial Strains

S. kasugaensis MB273 and its derivatives[10] (Table 1) were maintained on slants of ISP medium No. 2 (M2). Sl is a spontaneous aerial mycelium negative (Amy^-) mutant. S1A1 (Amy^-, Arg^-) is a mutant of S1 obtained by acriflavin (AF) treatment. A1R6 and A1R7 are Amy^+, Arg^+ revertants obtained by protoplast-regeneration of S1A1. R6N1 (Mal^-) and R6N2 (His^-) are mutants of A1R6 obtained by nitrosoguanidine (NTG) treatment. The procedures for AF and NTG treatment have been reported previously[11]. Auxotrophic mutants grown on M2 plates were detected by replica plating on MM plates (mannitol 1%, $(NH_4)_2SO_4$ 0.25%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.4%, trace element solution[12] 0.2% v/v, KH_2PO_4 0.2%, $Na_2HPO_4 \cdot 12H_2O$ 0.8%, agar 1.8%).

Table 1. *S. kasugaensis* MB273 derivatives and their plasmids.

Strain	Remarks	Plasmids	Source/Treatment
MB273	Parent	pSK1, pSK2, pSK3$^{(m)a)}$, pSK4$^{(t)b)}$	
189	Amy±	pSK1, pSK4$^{(t)}$	Stock culture[10]
18a	Amy$^-$, Arg$^-$	pSK3, pSK1$^{(t)}$, pSK2$^{(t)}$, pSK4$^{(t)}$	Stock culture[10]
R5	Amy$^-$	pSK2	Stock culture[10]
R11	Amy±	pSK1, pSK4$^{(m)}$	Stock culture[10]
S1	Amy$^-$	pSK1, pSK2, pSK3$^{(m)}$, pSK4$^{(t)}$	MB273/Spontaneous
S1A1	Amy$^-$, Arg$^-$	pSK1, pSK2, pSK3$^{(m)}$, pSK4$^{(t)}$	S1/Acriflavin
A1R2	Amy$^-$, Arg$^-$	A small amount of pSK1 and pSK2	S1A1/Protoplast
A1R21	Amy$^-$, Arg$^-$	Plasmid-free	A1R2/Protoplast
A1R6	Amy$^+$, Arg$^+$	Plasmid-free	S1A1/Protoplast
A1R7	Amy$^+$, Arg$^+$	pSK1*$^{c)}$	S1A1/Protoplast
R6N1	Amy$^+$, Mal$^-$	Plasmid-free	A1R6/NTG
R6N2	Amy$^+$, His$^-$	Plasmid-free	A1R6/NTG
R5I	Amy$^+$	pSK2*$^{d)}$	R5/Pock formation

Amy: aerial mycelium formation; Arg$^-$, His$^-$: requirement for argine and histidine, respectively; Mal$^-$: maltose utilization negative.

$^{a)}$ (m): a minor component, $^{b)}$ (t): a trace component, $^{c)}$ pSK1*: pSK1 having a pock-forming ability, $^{d)}$ pSK2*: pSK2 having a pock-forming ability.

Pock Formation

The donor and recipient strains were grown with shaking at 135 rpm and 28°C (the standard condition) for 40 hours in 3.0 ml of GlPYG medium (GPYG medium[10] with 0.3% peptone). *S. kasugaensis* MB273 grows as short hyphae resembling bacilli in liquid medium. Five-hundredth ml of a diluted culture of the donor strain (500~10,000 viable hyphae) was spread on M2 plates or GMY plates (MM in which mannitol was replaced by 0.5% glycerol and 0.05% yeast extract (Difco)) with or without a large excess of recipient cells (0.15 ml; approximately 5×10^8 viable hyphae) and incubated at 28°C for a week. Pocks became visible after 3 days of incubation. The frequency of pock formation was calculated as the number of pocks per number of donor colonies.

Preparation, Regeneration and Transformation of Protoplasts

Protoplasts of *S. kasugaensis* MB273-derivatives were prepared and regenerated by the procedure of SHIRAHAMA *et al.*[9] except that the mycelium was grown in GlPYG medium containing 1% glycine. The regeneration frequency was determined as described by SHIRAHAMA *et al.*[9].

Transformation was carried out by a modification of the procedure of BIBB *et al.*[13]. To a DNA sample (20 μl; 0.2 μg pSK1* DNA for the experiment of Fig. 6) in a sterile tube (5 ml), 5 μl of 50% sucrose solution, 80 μl of protoplast suspension (5×10^7) and 105 μl of 40% polyethylene glycol (PEG) (mol wt. 2,000; Wako Pure Chem. Ind., Ltd.) in PWP[9] solution were added sequentially and mixed. The mixture was maintained for 2 minutes at room temperature (Fig. 6) or at 0°C followed by addition of 1.7 ml of PWP solution and allowed to stand for 5 minutes at room temperature (Fig. 6) or at 0°C; three-tenth ml was transferred to R3 plates[9] and overlaid with 3.5 ml of soft R3-agar medium and allowed to regenerate for 2 weeks at 27°C[9]. The resultant colonies (very small colonies) failed to sporulate and to exhibit pocks. Percentage (%) of transformants among viable hyphae was determined after growth in a liquid culture as follows: about one-fourth of the regenerated colonies ($>10^4$) were scraped off a plate and inoculated into 3.0 ml of GlPYG medium and grown with shaking overnight; the mycelial clumps and agar from the plate were allowed to settle for 10 minutes; the hyphae in the liquid phase were analyzed for the frequency of pock formation as described above. The mean frequency from two studies for each transformation experiment was used as indicator of the transformability (%) of the protoplasts (Fig. 6).

DNA Preparation

Chromosomal DNA: *S. kasugaensis* chromosomal DNA was isolated from the plasmid-free strain

A1R6 by a modification of the method of MARMUR[14]. A1R6 was grown in 20 ml of GlPYG medium in a 100-ml flask with shaking for 40 hours. The mycelium (8-ml culture) was harvested and washed with 2× TES buffer[15] by centrifugation at 8,000 rpm and 4°C for 7 minutes. The mycelium, suspended in 10 ml of 2× TES buffer containing lysozyme (1 mg/ml), was incubated for 60 minutes at 37°C and the DNA recovered. The purified DNA was dissolved in DSB buffer[15] and dialyzed against the same buffer.

Plasmid DNA: Plasmid DNA was isolated on a small scale for screening purposes by a modification of the alkaline-extraction method described by BIRNBOIM and DOLY[16]. The test strains were cultured with shaking for 40 hours in 3.0 ml of GlPYG medium. The mycelium of each culture (two cultures in the case of preliminary screening of plasmids with deletions or insertions) was collected by centrifugation and treated with lysozyme (1 mg/ml) for 60 minutes at 33°C in 2.0 ml of SSCa solution (sucrose 17%, NaCl 0.4%, $CaCl_2 \cdot 12H_2O$ 0.15%, 0.25 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, pH 7.2, 10%). The mixture was centrifuged for 7 minutes at 8,000 rpm and 4°C, and the pellet was suspended in 0.3 ml of SSCa solution and then solubilized in 1.5 ml of alkaline-lytic solution (2× TES buffer containing 1% of sodium-*N*-lauroyl sarcosinate and NaOH, pH 12.9). After 10 minutes of incubation at pH 12.3 and room temperature, the pH of the sample was adjusted to pH 8.0 by addition of 0.5 ml of 2 M Tris-HCl buffer (pH 7.0). The crude lysate was digested with Pronase E (100 μg/ml; Kaken Seiyaku Co., Ltd.) for 60 minutes at 37°C and then centrifuged for 30 minutes at 16,000 rpm and 4°C. Plasmid DNAs in the cleared lysate (1~2 ml) were precipitated overnight at 4°C after addition of 5 M NaCl and 40% PEG (MW 7,500; Wako Pure Chem. Ind., Ltd.) in 2× TES buffer to give a final concentration of 0.5 M and 10%, respectively. The pellet collected by centrifugation for 7 minutes at 8,000 rpm and 4°C was dissolved in 0.2~0.3 ml of TES buffer and stored at 4°C. This preparation was subjected to agarose gel electrophoresis for detection of plasmids. The scaled-up procedure was also applied to extract from 100-ml cultures; 4.0 ml of the PEG preparation was obtained.

Purification of Plasmids

The preparation isolated from 100-ml cultures was dialyzed against TES buffer, then digested with RNase (100 μg/ml; Miles Lab (PTY) Ltd.) for 60 minutes at 37°C, treated twice with phenol saturated with 3% NaCl after addition of 30% NaCl to give a final concentration of 3%, and washed with a mixture of chloroform: isopentyl alcohol (24: 1). The plasmid DNA was purified by ethidium bromide CsCl-buoyant density gradient centrifugation for 42 hours at 38,000 rpm and 20°C and then dialyzed against DSB buffer.

Restriction Enzyme Digestion and Agarose Gel Electrophoresis (AGE)

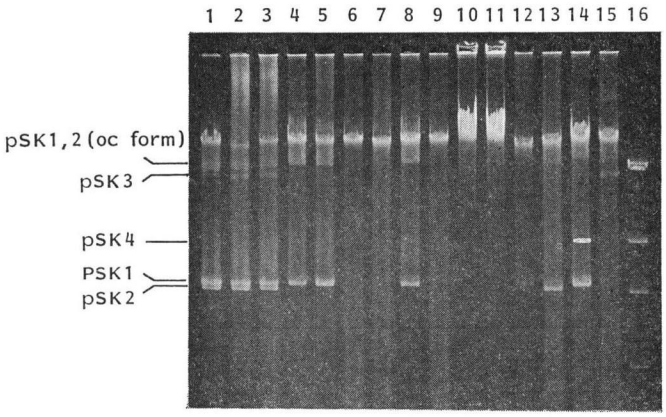
Restriction endonucleases, except for *Bcl* I, were purchased from Takara Shuzo Kaisha, Ltd. *Bcl* I was from Boehringer Mannheim GmbH. These were used as directed by the manufacturer. Electrophoresis was carried out using a horizontal slab gel consisting of 1% agarose (Seakem Agarose (ME)) in Tris-phosphate buffer (Trisma base 89 mM, EDTA-2Na 2.5 mM, H_3PO_4 23 mM, pH 8.3) at a constant voltage (100 V) for 1.5 hours or in TEAS buffer (Trisma base 50 mM, EDTA-2Na 2 mM, sodium acetate 20 mM, NaCl 18 mM, pH 8.1) at 70 V for 16 hours (Fig. 1). Molecular weights of DNA fragments were determined by comparison with a *Hin*d III digest of bacteriophage λ DNA.

Pock-Forming Derivatives of pSK1* and pSK2* with DNA Insertions or Deletions

The mixture of pSK1* (1 μg) and pSK2* (1 μg) was digested completely with *Bgl* II, *Bcl* I, *Bam* HI or *Eco* RI, respectively or digested partially with *Kpn* I or *Sac* I, respectively. The digests were treated with phenol and dialyzed against DSB buffer. Each digest (40 μg/ml) was heated for 7 minutes at 70°C and cooled gradually for 3 hours to 4°C and ligated for 20~40 hours at 4°C with T4 DNA ligase (Takara Shuzo Kaisha, Ltd.) in the recommended reaction mixture; ligation was monitored by AGE. A complete *Sal* I digest of pSK1* (0.5 μg) and a partial *Sal* I digest of the chromosomal DNA (4 μg) of *S. kasugaensis* MB273-A1R6 were mixed and ligated as described above. These ligated DNAs were used to transform protoplasts (5×10^7) of the plasmid-free strain A1R6, and the protoplasts were regenerated on R3 plates for 2 weeks at 27°C. Transformants were screened by pock formation from the regenerated-colonies as described in the above section. Strains isolated from

Fig. 1. Agarose gel electrophoresis of plasmids in crude extracts of *S. kasugaensis* MB273 and its derivatives.

1: MB273, 2: S1, 3: S1A1, 4: A1R7, 5: N1T1, 6: A1R6, 7: A1R21, 8: N2T1, 9: R6N1, 10: R6N2, 11: G3, 12: 189, 13: R5, 14: R11, 15: 18a, 16: λ DNA/*Hin*d III



pock-centers were screened for pSK1* and pSK2* with insertions or deletions by analysis of the plasmid DNA with AGE.

Results

Isolation of Plasmid-free Strains of *S. kasugaensis* MB273

A spontaneous Amy$^-$ mutant (S1) was selected from *S. kasugaensis* MB273. Certain colonies derived from the S1 mutant produced Arg$^-$ auxotrophs (S1A1) at very high frequency (more than 90%) after acriflavin treatment. The Amy$^-$, Arg$^-$ mutant (S1A1) and the MB273 parental strain carry four plasmids: pSK1 (7.2 Md), pSK2 (7.1 Md), pSK3 (14.3 Md) and pSK4 (8~10 Md) (Fig. 1). Two hundred colonies regenerated from S1A1 protoplasts were screened for a plasmid-free Amy$^-$, Arg$^-$ strain. Some contained reduced amounts of the plasmids (A1R2) and others, increased amounts of pSK3 (A1R3), but no plasmid-free strain was obtained. However, seven large colony variants with rough-type morphology were detected among the colonies regenerated from S1A1 protoplasts on R3 plates, that grew vigorously and sporulated on MM plates. Three of them (A1R6; Amy$^+$, Arg$^+$) were found to be plasmid-free, the others designated A1R7 (Amy$^+$, Arg$^+$) harbored only pSK1. Four plasmid-free Amy$^-$, Arg$^-$-strains (A1R21) were identified when one hundred colonies regenerated from A1R2 protoplasts were screened for the absence of plasmids; no Amy$^+$ or Arg$^+$ revertant was detected. AGE analysis of strain A1R6, A1R7 and A1R21 as well as other strains used in this study are shown in Fig. 1; the plasmid compositions of the strains are summarized in Table 1.

Pock Formation by *S. kasugaensis* MB273 Derivatives

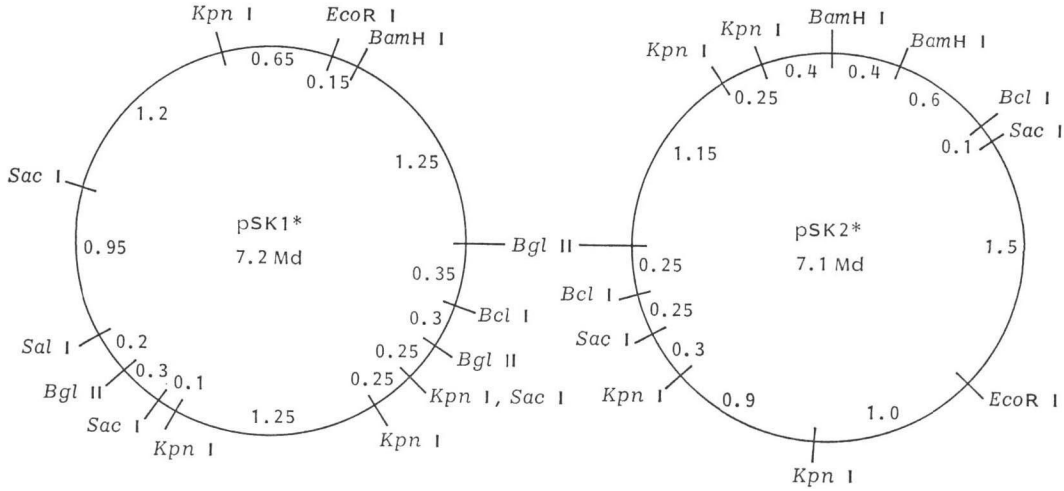
S. kasugaensis MB273 was found to cause pocks in a lawn of a plasmid-free strain of A1R6 on M2 plates. To establish which of the resident plasmids were involved in the pock formation, *S. kasugaensis* MB273 derivatives carrying various plasmids were tested for pock formation in a lawn of A1R6 (Table 2). MB273 and its Amy$^-$, Arg$^-$ mutant (S1A1) caused pocks at a frequency of 30%. Derivatives 189 (pSK1 and a small amount of pSK4) and 18a (increased amounts of pSK3 and reduced amounts of pSK1 and pSK2) caused pocks at approximately 0.1%, while derivative R5 (pSK2 alone) failed to

Table 2. Pock-forming ability of *S. kasugaensis* MB273 and its derivatives carrying plasmid(s) on plasmid-free *S. kasugaensis* MB273-A1R6.

Strain	Plasmids	Pocks/colonies tested (%) on M2 plate	Pocks/colonies tested (%) on GMY plate
MB273	pSK1, pSK2, pSK3$^{(m)a)}$, pSK4$^{(t)b)}$	30	50
S1A1	pSK1, pSK2, pSK3$^{(m)}$, pSK4$^{(t)}$	30	50
189	pSK1, pSK4$^{(t)}$	0.1$^{e)}$	0.2
R5	pSK2	<0.01	0.1$^{d)}$
18a	pSK3, pSK1$^{(t)}$, pSK2$^{(t)}$, pSK4$^{(t)}$	<0.1	0.1$^{e)}$
R11	pSK1, pSK4$^{(m)}$	1.4	7.5$^{e)}$
A1R7	pSK1*	100	100
R5I	pSK2*	100	100
N1T1$^{e)}$	pSK1*	100	100
R6N1	Plasmid-free	<0.0001	<0.0001

$^{a)}$ (m): a minor component, $^{b)}$ (t): a trace component, $^{e)}$ all the strains from the pocks (60/60) carried pSK1* alone, $^{d)}$ all the strains from the pocks (8/8) carried pSK2* alone, $^{e)}$ a strain isolated from the pock caused on a lawn of R6N1 (Mal$^-$) by a transformant of R6N1 with pSK1* DNA.

Fig. 2. Restriction-enzyme cleavage maps of pSK1* and pSK2*.



elicit the pock phenotype in the lawn on M2 plates. By contrast, A1R7, N1T1 (a strain isolated from a pock produced on a lawn of R6N1 (Mal$^-$) by a transformant of R6N1 with plasmid DNA from A1R7) and strains, which were isolated from the pocks caused by strains 189 and 18a, showed a pock-forming frequency of 100% and their plasmids were all identical to pSK1 in regard to molecular weight and endonuclease cleavage map. This plasmid was designated pSK1*. The restriction endonuclease cleavage map of pSK1* is shown in Fig. 2.

In the lawn of A1R6 on GMY agar medium, some strains showed enhanced frequency of pocking. For example, strain S1A1 (pSK1 ~ pSK4) caused pocks at a frequency of about 50% on GMY plates but only 30% on M2 plates; strain R5 (pSK2) caused 2 ~ 5 pocks per 10^4 on GMY plates and none on M2 plates (Table 2). The strain R5I, which was isolated from the center of pocks caused by strain R5 on a lawn of A1R6, caused a very high frequency of pocking on GMY plates (100% of the donor

Fig. 3. Pocks caused by *S. kasugaensis* MB273 derivatives carrying pSK1* (A1R7) or pSK2* (R5I) on *S. kasugaensis* MB273-A1R6.

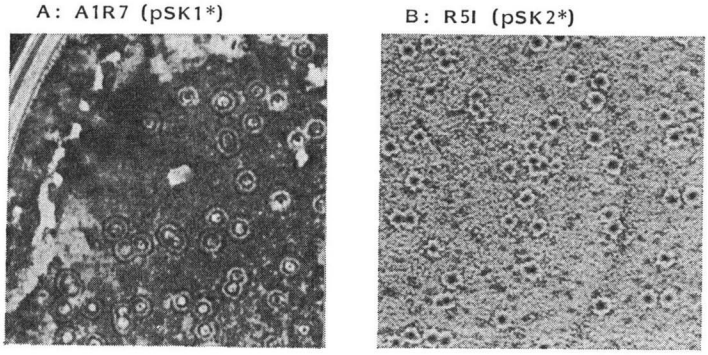


Table 3. Pock formation by *S. kasugaensis* MB273 derivatives carrying pSK1*, pSK2* and their derivatives.

Donor strain	Pock formation in the lawn of recipient strain					
	A1R6 (plasmid-free)	N1T1 (pSK1*)	N2C1 (pSK1*)	N2T2 (pSK2*)	R6T5 (pSK1*-1)	R6T6 (pSK2*-1)
N1T1 (Mal$^-$, pSK1*)[a]	+	−	−	+	−	+[f]
N2C1 (His$^-$, pSK1*)[b]	+	−	−	+	−	+[f]
N2T2 (His$^-$, pSK2*)[c]	+	+	+	−	+[f]	−
R6T5 (pSK1*-1)[d]	+	−	−	+[g]	−	+
R6T6 (pSK2*-1)[e]	+	+[f]	+[g]	−	+	−

[a] A strain isolated from a pock caused on a lawn of R6N1 (Mal$^-$) by a transformant of R6N1 with pSK1* DNA, [b] the recipient type of strain (His$^-$) isolated from a pock caused on a lawn of R6N2 (His$^-$) by a donor strain N1T1 (Mal$^-$, pSK1*), [c] a strain isolated from a pock caused on a lawn of R6N2 (His$^-$) by a transformant of R6N2 with pSK2* DNA, [d] a strain isolated from a pock caused on a lawn of A1R6 by a transformant of A1R6 with pSK1*-1 DNA, [e] a strain isolated from a pock caused on a lawn of A1R6 by a transformant of A1R6 with pSK2*-1 DNA, [f] a strain isolated from the pock yielded both plasmids of the donor and the recipient, [g] the recipient type of strain (His$^-$) isolated from the pock yielded both plasmids of the donor and the recipient.

Table 4. Pock-forming derivatives of pSK1* and pSK2* produced by deletion or insertion.

Derivative plasmid	Original plasmid	Inserted (or excised) DNA[a]	Ligation site(s)[b]
pSK1*-1	pSK1*	Chromosomal DNA; 1.5 Md	*Sal* I
pSK1*-2	pSK1*	pSK2* *Bcl* I A; 4.05 Md	*Bcl* I
pSK1*-3	pSK1*	pSK1* *Bgl* II C; 0.65 Md	*Bgl* II-b or -c
pSK1*-4	pSK1*	(pSK1* *Bgl* II C; 0.65 Md)	*Bgl* II-b and -c
pSK1*-5	pSK1*	pSK1* *Kpn* I D; 0.25 Md	*Kpn* I-c or -d
pSK1*-6	pSK1*	(pSK1* *Kpn* I D; 0.25 Md)	*Kpn* I-c and -d
pSK2*-1	pSK2*	pSK1* *Bgl* II C; 0.65 Md	*Bgl* II
pSK2*-2	pSK2*	pSK1* *Bgl* II B; 2.15 Md	*Bgl* II

[a] and [b] refer to Fig. 5.

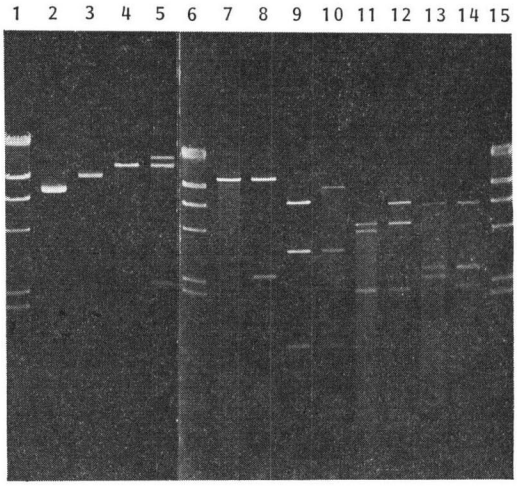
colonies). The plasmid isolated from strain R5I was found to be indistinguishable from pSK2 and was designated as pSK2* (Fig. 2). Strain R11 (pSK1 and pSK4) caused pocks at a low frequency (1.4~7.5%); colonies isolated from the center of pocks harbored pSK1* alone. No strains carrying pSK3 or pSK4 alone and exhibiting high frequency pocking have been isolated (Table 2).

Differences in pock morphology elicited by pSK1* and pSK2* were observed on lawns of strain A1R6 grown on GMY plates (Fig. 3). Pocks elicited by pSK1* appeared to possess alternate concentric rings of inhibition and growth surrounding a centrally-located minute colony, however pocks arising from strains bearing pSK2* displayed only a growth-inhibited region.

Pock-forming ability was tested using homologous and heterologous strains bearing the plasmids pSK1* or pSK2*, respectively. As shown in Table 3, a pock was seen only when the donor strain was tested on a plasmid-free strain or a strain carrying a heterologous plasmid.

Fig. 4. Agarose gel electrophoresis of digests of pSK1*-1 and pSK1*.

1: λ DNA/*Hin*d III, 2: pSK1* (ccc form), 3: pSK1*-1 (ccc form), 4: pSK1*/*Sal* I, 5: pSK1*-1/*Sal* I (patrial digestion), 6: λ DNA/*Hin*d III, 7: pSK1*/*Sal* I, 8: pSK1*-1/*Sal* I, 9: pSK1*/*Bgl* II, 10: pSK1*-1/*Bgl* II, 11: pSK1*/*Kpn* I, 12: pSK1*-1/*Kpn* I, 13: pSK1*/*Sac* I, 14: pSK1*-1/*Sac* I, 15: λ DNA/*Hin*d III.



Endonuclease Cleavage-Sites in pSK1* and pSK2* Nonessential for Pock Formation

Eight pock-forming derivatives of pSK1* and pSK2* have been obtained by excision or insertion of DNA fragments (Table 4). Four derivatives of pSK1* were found to have a DNA fragment at four different endonuclease cleavage-sites: *i.e.*, pSK1*-1, 1.5 Md, *Sal* I site (Fig. 4); pSK1*-2, 4.05 Md, *Bcl* I site (Fig. 5); pSK1*-3, 0.65 Md, *Bgl* II-b or *Bgl* II-c site (Fig. 5); pSK1*-5, 0.25 Md, *Kpn* I-c or *Kpn* I-d site (Fig. 5). Two derivatives of pSK1* had deletions from the *Bgl* II-b site to the *Bgl* II-c site (*Bgl* II-C) (pSK1*-4) or from the *Kpn* I-c site to the *Kpn* I-d site (*Kpn* I-D) (pSK1*-6), respectively (Fig. 5). Two derivatives of pSK2* (pSK2*-1, pSK2*-2) also had DNA inserts (0.65 Md, 2.15 Md) at the *Bgl* II site in pSK2*.

A donor strain carrying one of these derivative plasmids caused pocks on a lawn of a plasmid-free strain or a strain carrying a heterologous plasmid (Table 3; pSK1*-1, pSK2*-1 as examples). The strain isolated from the pock on the recipient strain carrying a heterologous plasmid contained both plasmids of donor and recipient strains, and moreover, the recipient type of strain (His^-) isolated from the pock was found to contain a plasmid of donor strain (pSK1*-1/or pSK2*-1) together with those of recipient one (pSK2*/ or pSK1*) (see legends f and g in Table 3).

Transformation of *S. kasugaensis* MB273 Derivatives

The effect of growth on efficient transformation and regeneration of protoplasts of *S. kasugaensis* MB273 was investigated using R6N1 (Mal^-) and R6N2 (His^-). The growth phase of the mycelium of strain R6N2 (His^-) was found to have a significant effect on the regeneration-frequency and transformability (its determination is described in Materials and Methods) of protoplasts as shown in Fig. 6. The mycelial mass attained a peak at 24 hours of growth and then decreased rapidly with further incubation. The decrease may be caused by cell lysis due to the high concentration of glycine (1%) in

Fig. 5. Agarose gel electrophoresis of endonuclease digests of pock-forming derivatives of pSK1* and pSK2* produced by deletion or insertion of DNA fragments.

1: pSK1*/*Bgl* II, 2: pSK1*-4/*Bgl* II, 3: pSK1*-2/*Bgl* II, 4: pSK2*-2/*Bgl* II, 5: pSK2*/*Bgl* II, 6: pSK1*/*Bcl* I, 7: pSK1*-2/*Bcl* I, 8: pSK2*/*Bcl* I, 9: pSK1*-6/*Kpn* I, 10: pSK1*/*Kpn* I.

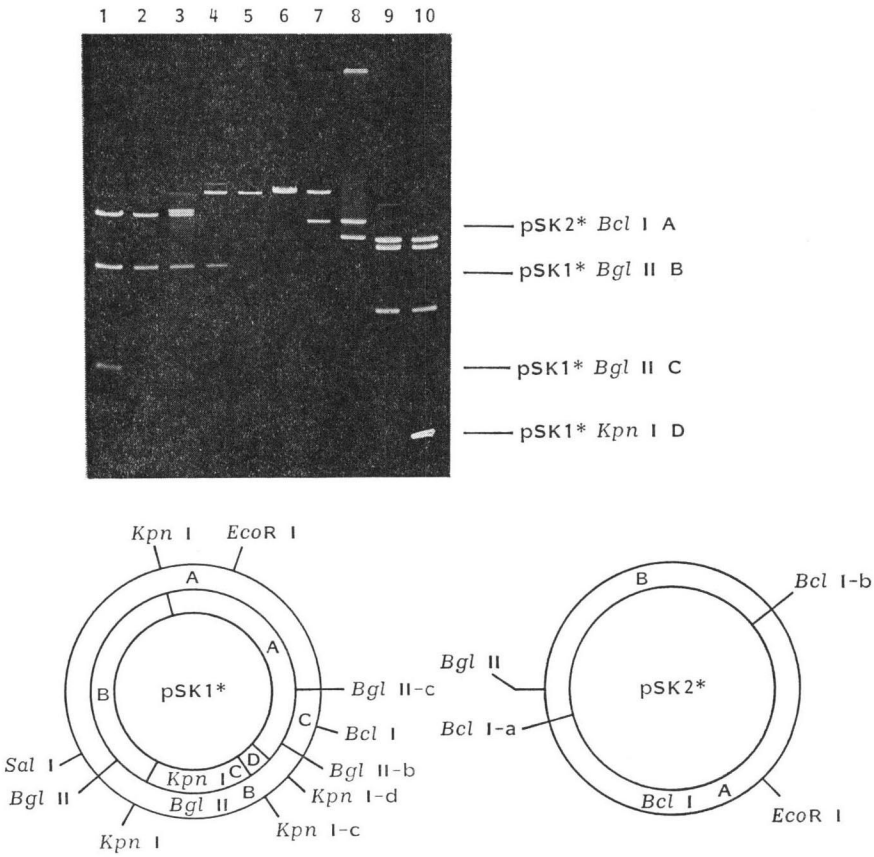
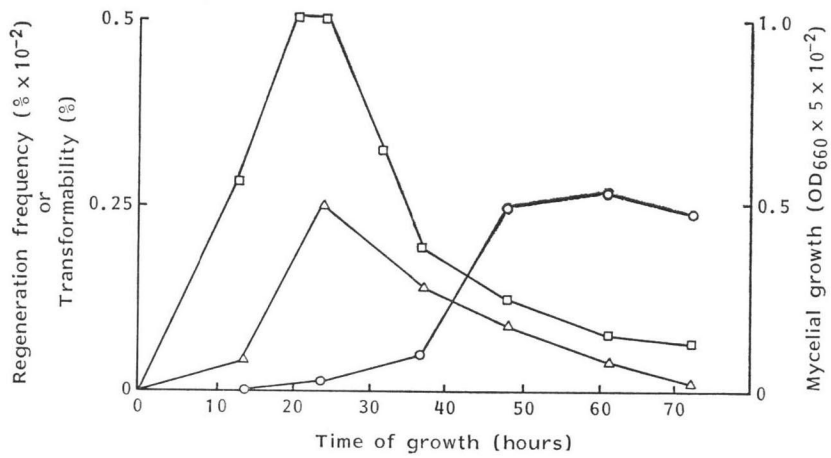


Fig. 6. Effect of age of culture of *S. kasugaensis* MB273-R6N2 on its protoplast-regeneration and transformation.

○ Transformability, △ regeneration frequency, □ mycelial growth.



the culture medium. Protoplast-regeneration frequency was directly correlated with growth phase of the organism. A rapidly growing culture (14 hours) yielded protoplasts of low viability (4.5% plating efficiency); however, after 24 hours cultivation of the organism protoplasts with the highest viability (25%) were obtained. Subsequently, protoplasts with progressively lower viability were obtained (14.5% at 37 hours and 1% at 72 hours). Protoplasts from 14 hours of 24 hours cultures were poorly transformed: in contrast, those from older culture (48 to 72 hours) were more efficiently transformed (Fig. 6). Similar results were obtained with protoplasts derived from strain R6N1 (Mal^-) and R6N2 (His^-). Transformation experiments carried out at 0°C increased the transformability of the protoplasts derived from 48 hours mycelium (1.0~1.2% vs. 0.18~0.27%, when 0.2 μg pSK1* DNA was used per 5×10^7 protoplasts).

Discussion

A plasmid-free strain of A1R6 of *S. kasugaensis* MB273 was obtained spontaneously from several Amy^+, Arg^+ revertants which appeared in the course of protoplast formation and regeneration of the Amy^-, Arg^- mutant. The mechanism of generation of such a revertant produced by protoplast formation and/or regeneration of the *S. kasugaensis* MB273 mutant is unknown. An additional plasmid-free strain A1R21 (Amy^-, Arg^-) was obtained from A1R2 (Amy^-, Arg^-, $plasmid^+$) by using the protoplast-regeneration procedure. These observations suggest that the process of protoplast formation and/or regeneration may play an role in plasmid elimination[10].

MB273 caused pocks on the plasmid-free A1R6 strain and the plasmids responsible for pock formation were examined. All MB273 derivatives carrying plasmid(s) were found to cause pock formation at high frequency (S1A1), low frequency (R11) or very low frequency (189, 18a, R5) (Table 2). On the other hand, strains A1R7 and R5I which harbor either pSK1* or pSK2*, and strains isolated from the center of pocks showed a very high frequency of pock formation (100% of the donor colonies). On the other hand, when plasmid DNA (0.2 μg) isolated from strains A1R7 (pSK1*) and R5I (pSK2*) was introduced into protoplasts of plasmid-free *S. kasugaensis* MB273-derivatives, pock-formable transformants were detected with about 1% frequency of viable hyphae obtained from the liquid culture after the regeneration; however pSK2 DNA (1 μg) isolated from strain R5 was ineffective. Plasmids isolated from strain A1R7 and R5I have been designated as pSK1* and pSK2*, respectively[1,17]. Evidence that pSK1* and pSK2* are distinct from each other is based on endonuclease cleavage maps (Fig. 2) and pock-forming capability (Table 3); *i.e.*, strains carrying pSK1* alone caused pocks on strains bearing pSK2* alone, and *vice versa*. Similar results have been observed for SCP1 and SCP2* in *S. coelicolor* A3(2)[17], for SLP1.2 and SLP4 in *S. lividans* 66[2] and for pIJ101 and SCP1, SCP2* or SLP-based plasmids in *S. lividans* ISP 5434[3]. The pock-forming plasmids SLP1.2[2] and pIJ101[3] were reported to carry determinant(s) for transfer and "spread" of the plasmids and for resistance to "lethal zygosis" elicited by a homologous plasmid. The regions of pSK1* and pSK2* responsible for transfer and resistance to "lethal zygosis" have not been established; however, pock formation in *S. kasugaensis* MB273-derivatives is the result of transfer of the transmissible plasmid pSK1* and pSK2* and of their derivative plasmids into recipient strains (Table 3); resistance to "lethal zygosis" applies only to a homologous plasmid (Table 3).

Several pock-forming derivatives of pSK1* and pSK2* have been obtained by excision or insertion of DNA fragments (Table 4). They include three derivatives with a DNA insert (1.5~4.05 Md) at single endonuclease cleavage-sites in pSK1* (*Bcl* I and *Sal* I) and in pSK2* (*Bgl* II), respectively. These endonuclease cleavage-sites are thus available for DNA cloning without destroying pock-forming ability.

An efficient transformation system for *S. kasugaensis* MB273 is useful for DNA cloning in this strain. THOMPSON *et al.*[18] pointed out the importance of the growth phase of *S. lividans* for efficient transformability and regeneration of protoplasts. The growth phase of *S. kasugaensis* MB273-deriva-

tives significantly affected both processes (Fig. 6): *i.e.*, protoplasts were competent for transformation after the stationary phase of mycelial growth, at which time their regeneration frequencies were decreasing. Moreover, transformation experiments carried out at 0°C were found to increase the transformability of protoplasts. These results suggest that the most efficient transformation frequency is attained when protoplasts derived from the mycelium grown for 48 hours were transformed at 0°C. This condition for transformation of protoplasts of *S. kasugaensis* MB273-derivatives may be useful for shot-gun cloning in this strain.

Acknowledgment

The authors express their deep gratitude to Dr. M. OKANISHI of our Department in National Institute of Health for his encouragement and advice throughout this work, and to Dr. H. UMEZAWA of Institute of Microbial Chemistry for his continuous interest and encouragement.

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